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Report

Regulation of Ras Signaling Dynamics by Sos-Mediated Positive Feedback

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Summary

The RTK-Ras-ERK cascade is a central signaling module implicated in the control of diverse biological processes including cell proliferation, differentiation, and survival. The coupling of RTK to Ras is mediated by the Ras-specific nucleotide-exchange factor Son of Sevenless (Sos), which activates Ras by inducing the exchange of GDP for GTP [1]. Considerable evidence indicates that the duration and amplitude of Ras signals are important determinants in controlling the biological outcome [2]. However, the mechanisms that regulate the quantitative output of Ras signaling remain poorly understood. We define a previously unrecognized regulatory component of the machinery that specifies the kinetic properties of signals propagated through the RTK-Ras-ERK cascade. We demonstrate that the establishment of a positive feedback loop involving Ras•GTP and Sos leads to an increase in the amplitude and duration of Ras activation in response to EGF stimulation. This effect is propagated to downstream elements of the pathway as reflected by sustained EGF-induced ERK phosphorylation and enhanced SRE-dependent transcription. As a consequence, the physiological endpoint of EGF action is switched from proliferation to differentiation. We propose that the engagement of Ras/Sos positive feedback loop may contribute to the mechanism by which ligand stimulation is coupled to discrete biological responses.

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Results and Discussion

Recent structure-function analyses of Sos have led to the identification of a previously unrecognized regulation mode of Sos-mediated guanine nucleotide exchange on Ras. In addition to its well characterized catalytic site which upon binding of nucleotide-loaded Ras promotes nucleotide displacement, Sos possesses a second Ras binding site that is distal to the catalytic site and interacts with nucleotide-bound Ras [3]. This interaction results in an increased affinity for Ras at the catalytic site and an increase in the enzymatic activity of Sos. Though both GDP- and GTP-bound Ras can allosterically stimulate Sos, GTP-bound Ras does so 10-fold more potently [4]. Because Ras•GTP is the product of the Ras-Sos exchange reaction in vivo, we have postulated that this allosteric effect may constitute a positive feedback mechanism for Ras activation where the product of the exchange reaction contributes to its further acceleration. In support of this idea, we have previously shown that Sos activity, both in vivo and in vitro, is greatly reduced when the binding of Ras to the distal site is compromised [4].

In an effort to further explore the functional significance of the allosteric regulation of Sos by Ras•GTP, we have sought to develop an experimental strategy that will permit the manipulation of Sos activity via the distal site in vivo. We have reasoned that this could be achieved by introducing into cells a Ras mutant that selectively interacts with the distal site. By necessity, such a mutant would have to display the following properties: (1) constitutive binding to GTP, (2) capacity to allosterically stimulate the exchange activity of Sos, and (3) a defect in downstream signaling. The third property would be critical for the ability to monitor Ras-dependent signals that are generated selectively through Ras that has been activated via Sos-mediated catalysis (Figure 1A). We have previously characterized a mutant of HRas, HRas A59G (hereafter referred to as Ras A59G), that displays two of these three properties, namely constitutive binding to GTP because of an impaired GTPase activity and the capacity to allosterically stimulate Sos [3, 5]. Moreover, when bound to GTP, this mutant is defective in Sos-mediated exchange and thus makes it a highly effective allosteric modulator of Sos [5, 6] (see Figure S1A in the Supplemental Data available with this article online).

By using Ras A59G as a template, we have searched for an intragenic mutation that would abrogate the Raf-1 effector interaction that is necessary for signaling through the ERK cascade without affecting Ras's ability to allosterically stimulate Sos activity. An aspartic acid at position 38 of Ras has been implicated in mediating interactions with Raf-1 [7, 8]. As illustrated in Figure 1B, substitution of this aspartic-acid residue with glutamic acid (Ras A59G,D38E) resulted in a 100-fold decrease in Raf-1 binding in vitro. This mutation did not alter the guanine nucleotide exchange and GTPase activities of

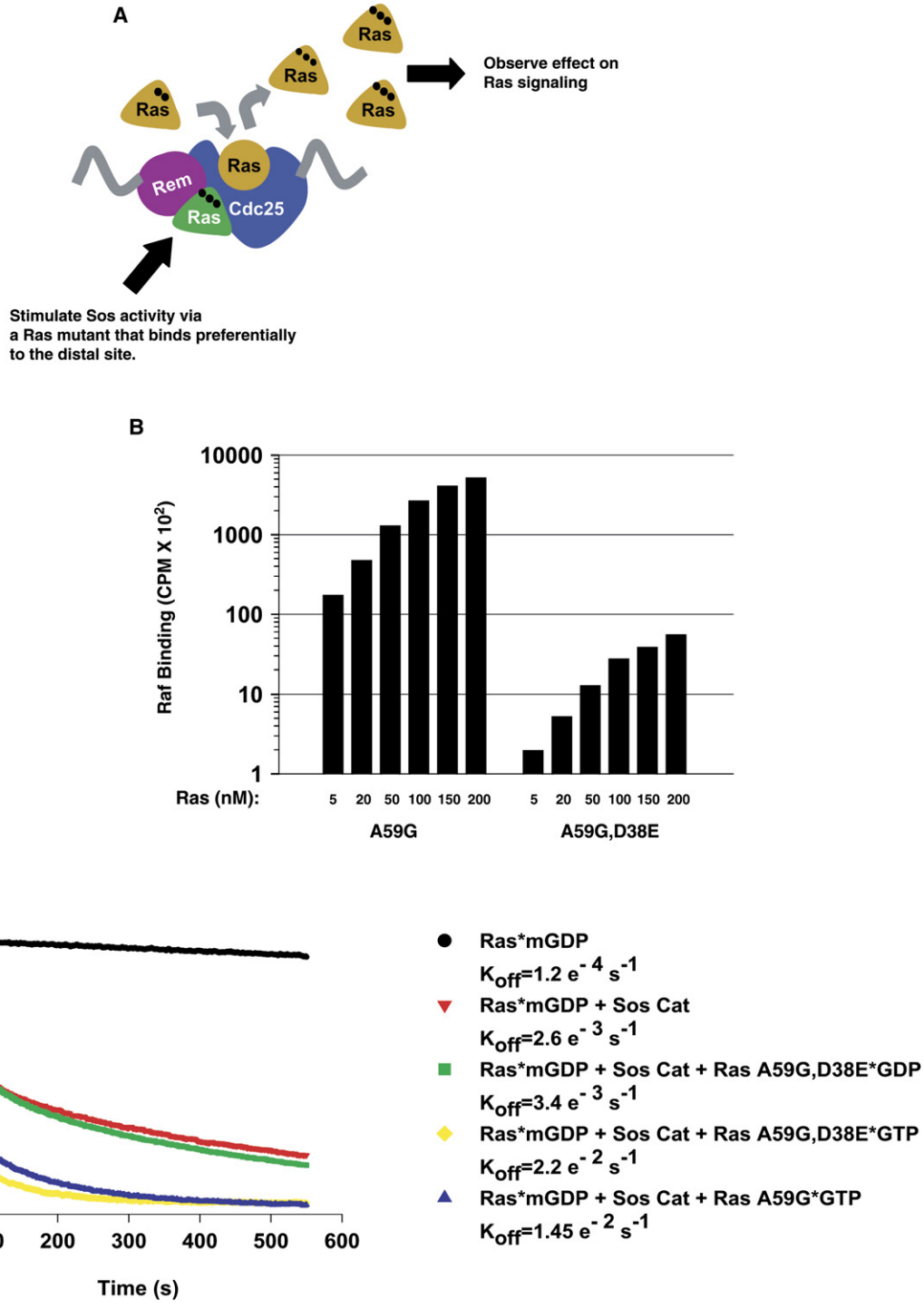

Figure 1. Biochemical Characteristics of Ras A59G,D38E

(A) A schematic illustration of the experimental design. The guanine-nucleotide-exchange activity of Sos is mediated by two structural elements: the Ras exchange motif (Rem) and the Cdc25 domain [25]. The catalytic site of Sos is contained within the Cdc25 domain. Allosteric regulation of Sos occurs through the distal site that is formed by the Rem and Cdc25 domain. A Ras mutant (green) that binds preferentially to the distal site of Sos is used to determine how allosteric modulation of Sos affects Ras signaling.

(B) Ras A59G,D38E has reduced affinity for the Ras binding domain (RBD) of Raf. An in vitro GST-Raf-RBD pull-down assay was performed with purified Ras A59G or Ras A59G,D38E loaded with ^{32}P-labeled GTP as described in the Supplemental Experimental Procedures. The GST-Raf-RBD was immobilized on glutathione agarose and incubated with indicated concentrations of Ras. At the end of the incubation period, the beads were washed and bound radioactivity was measured by scintillation counting.

(C) Ras A59G,D38E•GTP enhances Sos-Cat-catalyzed nucleotide-exchange activity. Comparison of intrinsic and Sos-Cat (1 μM)-catalyzed nucleotide exchange of mant-GDP for cold GDP from RasWT (1 μM) in the presence of stoichiometric amounts of either Ras A59G loaded with GTP or Ras A59G,D38E loaded with GTP or GDP. Nucleotide-exchange reactions were conducted as described in Supplemental Experimental Procedures. Rates were fitted to single exponentials. For (B) and (C), the results shown are representative of three independent experiments.

Ras A59G (Figures S1A–S1C). To test the capacity of Ras A59G,D38E to allosterically enhance Sos activity, we performed in vitro nucleotide-exchange assays by using the catalytic domain of Sos, hereafter referred to as Sos Cat. This domain spans residues 564–1049 and encompassed both the catalytic and distal Ras-binding sites. The rate of Sos-Cat-catalyzed nucleotide dissociation was significantly enhanced by Ras A59G,D38E•GTP to the same extent as the acceleration of nucleotide exchange observed with Ras A59G (Figure 1C). That this effect is exerted through the interaction of Ras A59G, D38E•GTP with the distal site is indicated by the fact that the GDP-bound form of Ras A59G,D38E had no affect on the kinetics of the exchange reaction under the conditions used here. Together, these results indicate that the D38E mutation does not interfere with Ras-mediated allosteric regulation of Sos. This conclusion is in agreement with structural data showing that aspartic acid 38 is not engaged in interactions that are critical for the binding of Ras to the distal site [3].

To ascertain that the biochemical properties of Ras A59G,D38E displayed in vitro are maintained in vivo, we first monitored by thin-layer chromatography the nucleotide-binding status of Ras A59G,D38E isolated from serum-starved cells. As illustrated in Figure 2A, we have found that Ras A59G,D38E exists in the cell predominantly in a GTP-bound form. To determine whether Ras A59G,D38E can enhance Sos activity in vivo through an interaction with the distal site, we cotransfected serum starved cells with Sos Cat and Ras A59G,D38E and used the phosphorylation of ERK mitogen-activated protein kinase (MAPK) as a readout for Ras activation [9, 10]. It should be noted that for these experiments, the levels of ectopically expressed Sos Cat were titrated down so that the detection of a potentiating effect could be facilitated. As demonstrated in Figure 2B, the expression of Ras A59G,D38E alone had no effect on ERK activation, consistent with the loss of Raf-1 binding observed for this mutant in vitro (Figure 1B). In contrast, in the presence of Sos Cat, the expression of Ras A59G,D38E led to an increase in ERK MAPK activation. The increase was abolished when Ras A59G,D38E was coexpressed with a previously characterized Sos Cat mutant that contains distal-site substitutions (Sos Cat L687E/R688A), which inhibit the binding of Ras•GTP [4]. These data indicate that Ras A59G,D38E enhances Sos activity in a manner that is dependent on the distal site. Hence, we refer to this mutant from here on as Enhancer Ras. Given that the occupation of the distal site by Ras•GTP mediates the formation of a positive feedback loop for Sos-dependent Ras activation, Enhancer Ras provides a means by which the function of such a feedback loop can be assessed in vivo.

It is well documented that in response to growth-factor stimulation, Ras undergoes a transient activation, the kinetics of which is growth-factor and cell-type dependent [11]. To investigate the effects of Enhancer Ras on growth factor-induced Ras activation, we cotransfected HeLa cells with HA-tagged wild-type Ras with or without T7-tagged Enhancer Ras, and measured the activation of Ras after epidermal growth-factor (EGF) stimulation by using a Raf-Ras-binding domain (RBD)

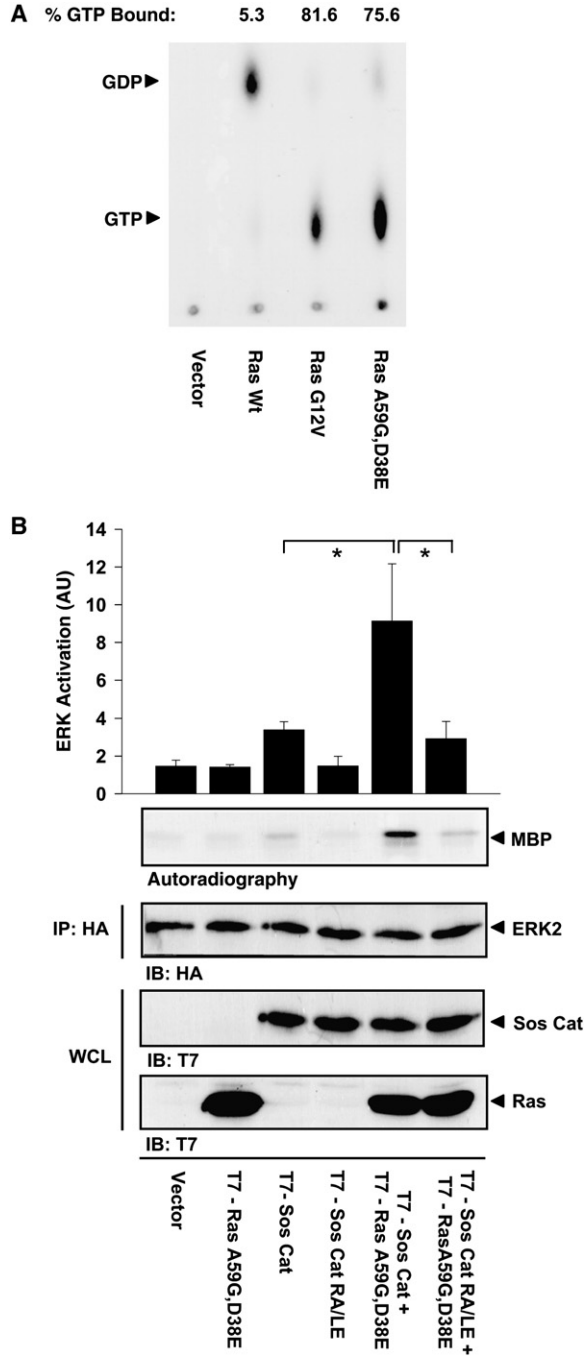

Figure 2. Biochemical Properties of Ras A59G,D38E In Vivo

(A) Ras A59G,D38E is constitutively active in serum-starved COS1 cells. COS1 cells were transfected with the indicated Ras expression vectors. The cells were serum-starved and labeled with [^{32}P]orthophosphate, and the guanine nucleotide content of the Ras proteins was analyzed by thin-layer chromatography as described previously [26]. The result is representative of two independent experiments.

(B) Ras A59G,D38E enhances Sos Cat activity in vivo. COS1 cells were cotransfected with HA-tagged ERK MAP kinase and the indicated constructs. The cells were subsequently serum-starved and ERK activation was determined by immunecomplex kinase assay as previously described [4]. Results shown represent the averages of four independent experiments. Error bars represent the standard deviations. *$p < 0.05$ (AU; arbitrary units).

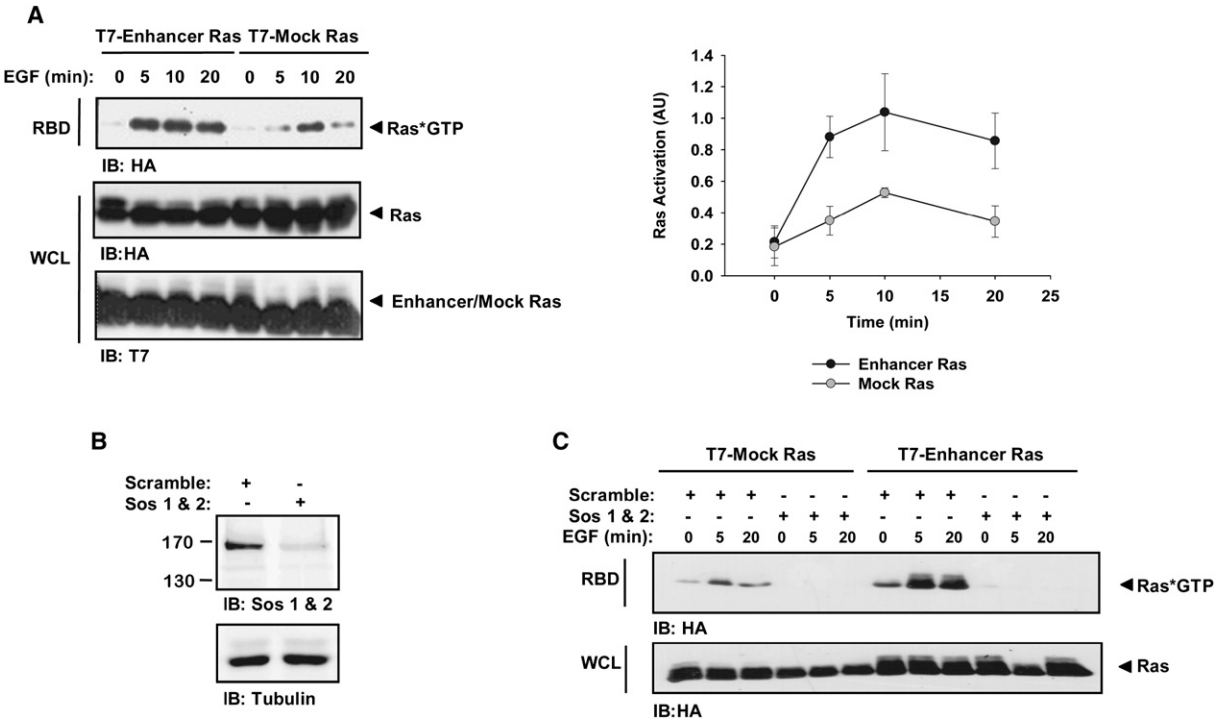

Figure 3. Allosteric Stimulation of Sos Influences the Kinetics of EGF-Induced Ras Activation

(A) Expression of Enhancer Ras results in an increased amplitude and duration of Ras activation in response to EGF. HeLa cells were cotransfected with HA-tagged RasWT and either T7-Enhancer Ras or Mock Ras. The cells were serum-starved and stimulated with EGF for the indicated intervals. The levels of GTP-bound RasWT were determined by pull-down assay with GST-Raf RBD as described in the Supplemental Experimental Procedures. The levels of activated RasWT were quantified by densitometry scanning and normalized to the levels of total RasWT. Results shown represent the averages of three independent experiments. Error bars represent the standard deviations. (AU; arbitrary units).

(B) Knockdown of Sos 1 and 2 by RNA interference. HeLa cells were transfected with pSuper vector containing Blasticidin resistance marker and targeting sequences for Sos 1 and 2 or scramble sequence. The cells were selected with Blasticidin prior to lysis. The levels of Sos were determined by immunoblotting of whole-cell lysate with an antibody that recognizes both Sos 1 and 2.

(C) The potentiation of Ras activation by Enhancer Ras is mediated by Sos. HeLa cells were cotransfected with indicated T7-tagged Ras constructs and HA-tagged RasWT along with pSuper constructs containing the Blasticidin resistance marker and the indicated target sequences. After selection with Blasticidin, the cells were serum-starved and then stimulated with EGF for the indicated intervals and lysed. The level of RasWT activation was determined as described in (A). For (B) and (C), the experiment shown is representative of three independent experiments.

pull-down assay [12]. As a control (Mock Ras), we have used a T7-tagged HRas mutant, HRas G12V,T35A, which is defective in effector interactions as well as in the ability to stimulate Sos catalytic activity (data not shown [13, 14]). Consistent with earlier studies, we have found that the levels of active Ras reached a peak within 10 min of EGF stimulation and declined to basal levels by 20 min (Figure 3A) [11]. In the presence of Enhancer Ras, Ras activation was approximately 3-fold higher, reached maximum within 5 min of EGF stimulation and remained at this level for up to 20 min (Figure 3A), and ultimately subsided at 40 min after stimulation (data not shown). These observations indicate that the kinetics of EGF-induced Ras activation can be modulated by Enhancer Ras both with respect to amplitude and duration.

To demonstrate that the effects of Enhancer Ras are mediated through its interaction with Sos, we have performed the same experiment by using HeLa cells in which Sos protein expression was knocked down by RNA interference (RNAi). As illustrated in Figure 3B, the target sequence we have used resulted in a significant suppression (>85%) of the expression of both human Sos proteins (Sos1 and Sos2). Under these conditions, EGF-induced activation of Ras in the presence or absence of Enhancer Ras was abolished, indicating that the function of Enhancer Ras is Sos dependent (Figure 3C). It is of interest to note that the potentiating effect of Enhancer Ras is manifested only in the presence of EGF. This is likely because Sos remains autoinhibited in the absence of growth-factor signaling. The autoinhibited state of Sos has been shown to be conferred by the interaction of the Dbl homology (DH) domain of Sos with the distal site, and such interaction occludes the binding of Ras to this site [4]. The molecular mechanism mediating the relief of Sos autoinhibition remains to be established.

The Ras to ERK MAPK signaling cascade plays a central role in mediating a wide spectrum of receptor tyrosine kinase (RTK)-dependent cellular responses [15]. To assess whether the engagement of the distal site of Sos affects Ras to ERK MAPK signaling, we cotransfected HeLa cells with HA-tagged ERK and T7-tagged Enhancer Ras or Mock Ras as a control. ERK MAPK activation in response to EGF stimulation was monitored by probing of HA immunoprecipitates with phosphorylation-specific anti-ERK MAPK antibodies. Expression of Enhancer Ras resulted in a significant increase in the

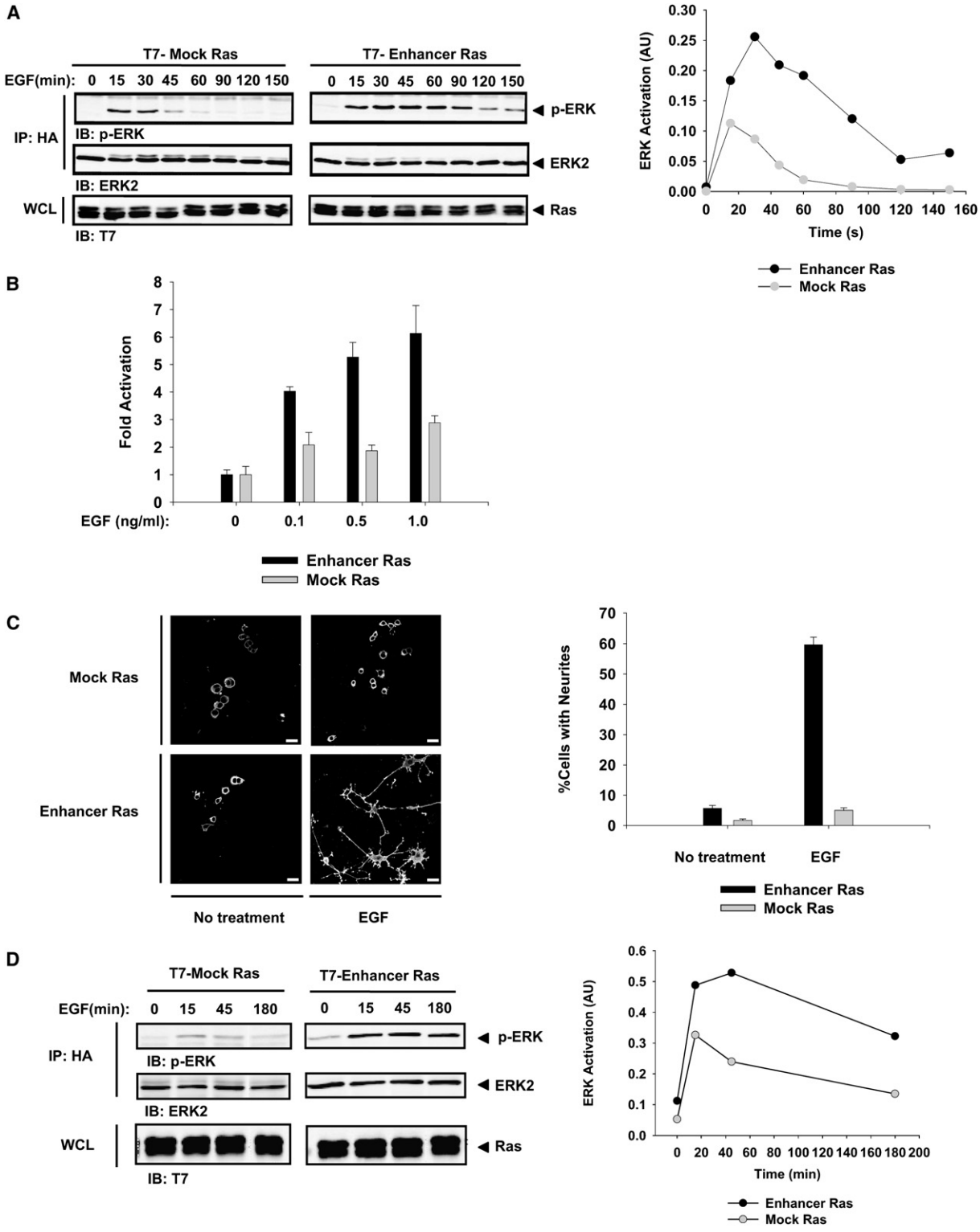

Figure 4. Contribution of Allosteric Stimulation of Sos to Signal-Transduction Events Downstream of Ras

(A) Expression of Enhancer Ras results in an increase in amplitude and duration of EGF-induced ERK MAP kinase activation. HeLa cells were cotransfected with the indicated T7-tagged Ras and HA-tagged ERK MAP kinase constructs. The cells were serum-starved and stimulated with EGF for the indicated intervals. ERK MAP kinase activation was determined by HA immunoprecipitation followed by immunoblotting with antibodies specific for ERK2 and phosphorylated ERK1/2 as described in the Supplemental Experimental Procedures. The levels of ERK activation, as represented by ERK phosphorylation, was quantified by densitometry and normalized to levels of total ERK2. The experiment shown is representative of three independent experiments. (AU; arbitrary units).

(B) Expression of Enhancer Ras sensitizes cells to growth-factor-induced transcriptional activation. HeLa cells were cotransfected with the indicated Ras constructs, SRE-Luciferase and CMV-β-Galactosidase (β-Gal). The cells were serum-starved and stimulated with the indicated

amplitude and duration of ERK MAPK activation in response to EGF compared with that in control cells (Figure 4A). RNAi-mediated knockdown of Sos resulted in an abrogation of EGF-induced ERK MAPK activation in the presence of Enhancer Ras (Figure S2), indicating that similarly to its effect on Ras activation, the effect of Enhancer Ras on ERK activation is Sos dependent. Collectively, these results implicate the distal site of Sos in the regulation of the dynamics of Ras signaling.

The conversion of transient activation of signaling pathways to long-term changes in cellular behavior is accomplished through changes in gene-expression patterns [16]. The serum response element (SRE) is a well-established transcriptional target of the Ras to ERK MAPK cascade [17]. To examine whether the transcriptional upregulation of SRE is influenced by the allosteric modulation of Sos, we cotransfected HeLa cells with either Mock or Enhancer Ras along with SRE-Luciferase and CMV-β-Galactosidase (β-Gal) and assayed them for the induction of luciferase activity in response to increasing concentrations of EGF. As shown in Figure 4B, SRE activity in response to EGF was augmented in cells expressing Enhancer Ras relative to cells expressing Mock Ras. Collectively, the potentiating effects of Enhancer Ras on EGF-induced Ras activation, ERK phosphorylation and SRE-dependent transcription indicate a role for Sos-mediated positive feedback of Ras activation in dictating the signaling output of the Ras pathway.

It has been shown that differences in the kinetics of ERK activation can be coupled to specific biological outcomes. For example, in PC12 cells, nerve growth factor (NGF) induces sustained activation of ERK and promotes differentiation into sympathetic-like neurons. In contrast, EGF induces transient ERK activation and cell proliferation [16]. Because EGF-induced ERK activation is prolonged in the presence of Enhancer Ras (Figure 4A), PC12 cell differentiation provides an effective experimental system for assessment of the extent to which Sos-mediated positive feedback regulation of Ras can contribute to the specification of a physiological process. To address this question, we cotransfected PC12 cells with T7-tagged Enhancer Ras or Mock Ras and treated them with EGF for 72 hr. Cells expressing T7-tagged Ras were detected by indirect immunofluorescence, and differentiation was assessed by visualization of neurite extension. As illustrated in Figure 4C, 60% of the Enhancer Ras expressing cells underwent differentiation, whereas the same treatment had no effect on cells expressing Mock Ras. Consistent with this effect, the expression of Enhancer Ras led to an increase in the intensity and duration of EGF-induced Ras and ERK activation (Figure 4D and Figure S3). The Mock Ras expressing cells retained the ability to undergo differentiation in response to NGF (data not shown). Thus, the catalytic potential of Sos may be an important determinant in specifying discrete cellular responses. It should be noted that the present study has relied primarily on ectopic-expression approaches to manipulate Sos activity. The development of pharmacological tools that target specific elements of the Sos-mediated exchange reaction in vivo may provide further insights into the link between the output of this reaction and the physiological outcome.

Several mechanisms have been implicated in the regulation of the temporal pattern of ligand-induced activation of the Ras to ERK cascade in mammalian cells. These include the differential recruitment of adaptor molecules to activated receptors [18–20] as well as differences in the surface density or intracellular trafficking fate of receptors [21]. In addition, a positive feedback loop between ERK and PKC signaling and a negative feedback loop set up by the transcriptional upregulation of Sprouty have been shown to influence the dynamic of the Ras to ERK cascade [22, 23]. The findings presented in this study identify Sos as a participant in the regulatory network that controls the quantitative output of the Ras pathway. The role of Sos in this context depends on its interaction with the product of the reaction it catalyzes, Ras•GTP, and the positive feedback loop that is generated by this interaction can prolong and amplify the response to a signal. Positive feedback is a key regulatory modality by which signaling systems are kinetically shaped with respect to bistability, inducibility, and resistance to noise [24]. The placement of such regulatory mechanism at the apex of the Ras signaling cascade may serve to ensure that the functional output of the pathway is aligned with a context-mandated cellular response.

Supplemental Data

Supplemental Data include Experimental Procedures and three figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/21/2173/DC1/.

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concentrations of EGF. The cells were lysed, and luciferase activities were measured and normalized for transfection efficiency with β-Galactosidase as described in the Supplemental Experimental Procedures. Results shown represent the average of three independent experiments. Error bars represent the standard deviations.

(C) Expression of Enhancer Ras promotes neurite extension in PC12 cells in response to EGF. PC12 cells were transfected with T7-tagged Mock Ras or Enhancer Ras, incubated with or without EGF for 48 hr, and subsequently fixed. The cells expressing Mock or Enhancer Ras were detected by indirect immunofluorescence staining with T7 antibody and scored for neurite outgrowth when their neurites extended beyond the length of two cell-body diameters. Representative fluorescence micrographs for each condition are shown. Scale bars represent 20 μm. Results shown represent the averages of three independent experiments. Neurite outgrowth was expressed as the percentage of T7-tagged Ras expressing cells with neurite extension. Error bars represent the standard deviations.

(D) Expression of Enhancer Ras results in an increase in amplitude and duration of EGF-induced ERK MAP kinase activation in PC12 cells. PC12 cells were cotransfected with the indicated T7-tagged Ras and HA-tagged ERK MAP kinase constructs. The cells were serum-starved and stimulated with EGF for the indicated intervals. ERK MAP kinase activation was determined as described in (A). The experiment shown is representative of three independent experiments. (AU; arbitrary units).

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